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Cloning of Flavin Reductase into pET32a(+) Expression Vector Lacking the Thioredoxin A Tag to Study Solubility of EDTA Monooxygenase A in Overexpression Systems

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The formation of inclusion bodies is a major obstacle for getting efficient bacterial protein production in expression systems. The pET32a(+) expression vector from Novagen fuses protein of interest to thioredoxin A (TrxA) to help facilitate disulfide bond formation in the cytoplasm greatly reducing protein aggregates. Flavin oxidoreductase (Fre) is another thioredoxin enzyme and may present another option. An attempt was made to clone the PCR amplified *fre* gene into pET32a(+) that had the thioredoxin A (TrxA) tag removed. Since it is unknown how the redox state of thioredoxin enzymes are able to improve protein solubility we wanted to compare expression of the highly insoluble protein EDTA monooxygenase A (EmoA) as a fusion protein to Fre and as independent co-expression. The project was delayed at the cloning step due to the difficulty of working with the NdeI restriction enzyme. It was found that a ligation condition with a low amount of T4 DNA ligase improve the poor ligation efficiency of NdeI digested fragments. A re-circularized vector with the TrxA tag removed was made as well as three potential clones that may contain the Fre insert. Attempts to clone EDTA monooxygenase A into the new vector resulted in no clones.

Protein expression using bacterial systems have many advantages, such as rapid growth rate, ease of manipulation, and the ability to produce milligram quantities of proteins. A major caveat of the system is the formation of highly insoluble inclusion bodies in the cytoplasm resulting in non-functional protein products. To overcome this, proteins are expressed fused to a redox enzyme, such as thioredoxin A (TrxA). Thioredoxin are small enzymes that undergo redox reactions through the reversible oxidation of two cysteine thiol groups to a disulphide resulting in the transfer of two electrons and two protons. Protein fusion with TrxA lead to increased disulfide bond formation, decreasing the formation of proteins aggregates. Many have used this strategy, for example the catalytic domain of human 11-β-hydroxysteroid dehydrogenase type 1(11β-HSD1), a membrane bound protein, when expressed fused to TrxA was found to be functionally active (3). Banerjee *et al*. also reported the ability to produce functionally active scorpion neurotoxin Lqq-V in *Escherichia coli*. Both of these proteins contain many disulfide bonds in their structure and if expressed without TrxA would have formed highly insoluble aggregates preventing any expression studies to be carried out.

The mechanism as to how thioredoxin stimulate disulfide bond formation between two protein cysteine residues within the reducing environment of bacterial cytoplasm has been studied and elucidated; however, the extent to which protein solubility is improved is yet unknown. It is known in *E. coli* that a loss of function mutation in thioredoxin reductase (TrxB) no longer permits TrxA to carry out its reducing reactions leading to a less reduced environment in the cytpolasm. The commercially available pET32a(+) vector from Novagen permits genes of interest to be fused to *trxA* for high levels of expression with good solubility.

Flavin reductase (Fre) is another redox enzyme with similar catalytic reduction abilities as TrxA. In this study, we attempt to clone *fre* into an expression vector and determine if it is able to increase the solubility of overexpressed proteins. EDTA monooxygenase A (EmoA), a highly insoluble protein when overexpressed, was chosen to investigate the effects of the redox enzyme in reducing the formation of protein aggregates in overexpression system (2). More specifically, we want to determine if the increase in protein solubility is due to the reductase enzyme activity or due to fusion protein properties. A construct of pET32a(+) vector with its *trxA* tag removed was made. Attempts were made to clone *fre* into the recircularized expression vector resulting in six potential clones.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. The bacterial strain and plasmids used in this study are shown in Table 1. *E. coli* DH5α were grown in Luria-Bertani (LB) broth (10g/L tryptone,

Table 1. Bacterial strains and plasmids

Strain or plasmid		Source or reference
E. coli		
DH5α	Host for plasmid amplification	UBC
DH5α: SW051 to SW056	pET32a(+)?*trxA/fre*$^+$, *lacI*, Amp^r_{100}	This study
DH5α: SW057	pET32a(+)?*trxA*, *lacI*, Amp^r_{100}	
Plasmid		
pET32a(+)	*trxA*, *lacI*, Amp^r_{100}	Novagen (6)
pES1	*fre* in pET30 LIC vector, Kan^r_{50}	(9)
pEmoA	*emoA* in pYrc99A, Amp^r_{100}	UBC
pET32a(+)?*trxA*	pET32a(+) with *trxA* removed	This study
pET32a(+)?*trxA/fre*$^+$	pET32a(+)?*trxA* with *fre* inserted	This study
pET32a(+)?*trxA/emoA*$^+$	pET32a(+)?*trxA* with *emoA* inserted	This study

[a] Amp^r_{100}, ampicillin at 100 µg/mL; Kan^r_{50}, kanamycin at 50 µg/mL.

5g/L yeast extract, 10g/L sodium chloride) or on LB agar (LB broth with 15g/L agar) supplemented with the appropriate antibiotics. The cultures were incubated at 37 °C and grown overnight with or without shaking 150 rpm. Purified plasmids were isolated using the standard protocol from the QIAprep spin miniprep kit (Qiagen, Cat.# 27104).

Plasmid transformation into DH5α was achieved by both the calcium chloride and electroporation methods. The chemical competent cells were provided and prepared as described by Kazem (5), whilst the electrocompetent cells were supplied and made by Kartono (4).

Construction of pET32a(+)Δ*trxA*. To remove *trxA*, which is flanked by NdeI sites, 5 µg of pET32a(+) was digested with NdeI (Fermentas Life Sciences, Cat.# ER0582) and 1X Buffer R (Fermentas Life Sciences, Cat.# BR5) for 120 minutes at 37°C followed by heat inactivation for 20 minutes at 65°C. The digested product was electrophoresed through a 1% low melting point agarose gel (Invitrogen, Cat.# 15517-022) with 1X Tris-Borate-EDTA (1X TBE) at 100V for 60 minutes. A 5600 bp fragment was excised and gel purified using the standard protocols, with some deviations, from the QIAquick gel extraction kit (Qiagen, Cat.# 28704) and the Agarase method (Fermentas Life Sciences, Cat.# E00461). Deviations are as follow, after the removal of Buffer PE the QIAquick column, containing bound DNA, was heated at 65°C for 2 minutes to ensure that all residual ethanol was eliminated. Buffer EB was warmed to 65°C and the column was eluted with 50 uL three times. As for the Agarase kit, melting of agarose at 70°C was performed for 30 minutes and incubation with Agarase at 42°C was extended to 1 hour. The changes in protocols were necessary to increase DNA yields from the columns and to obtain complete melting of the low melting point agarose. Next, 0.1 µg and 1 µg of the purified expression vector with *trxA* removed was re-circularized using 0.1 U of T4 DNA ligase (Invitrogen and Cat.# 15224-041) at 16°C overnight. The ligation products were then resolved on a 1% agarose gel with 1X TBE, as well as cleaned up by performing a phenol/chloroform DNA extraction (7) to remove any salts and to concentrate the DNA for electroporation into DH5α cells.

PCR Amplification of *fre* from pES1. The 790 bp *fre* fragment was amplified from pES1 using forward and reverse primers that had been designed by Kazem (5) and Kartono (4). In short, the forward primer (*fre* forward 5'– TAG-GGG-AAT-TGT-GAG-CGG-ATA-ACA – 3') is complementary to the translational start site, whereas the reverse primer (*fre* reverse 5'– GGG-TTT-TTT-CAT-ATG-TCC-GAT-AAA-TGC – 3') contains mutations (underlined) to introduce an NdeI site and to change the *fre* stop codon into a glycine residue for production of fusion proteins. PCR mixture and conditions used were as described by Kazem (5) using 0.2 µg of uncut pES1.

Construction of pET32a(+)Δ*trxA/fre*$^+$. The PCR amplified *fre* product was resolved on a 1% low melting point agarose gel in 0.5X TBE and a 790 bp band was excised, gel purified, and digested with NdeI (Fermentas Life Sciences, Cat.# ER0582). Several ligation reactions were set up as outlined in Table 2 with molar ratios of insert:vector of 3:1 and 9:3. Various ligation conditions were attempted using the

Table 2. Conditions for ligation reactions of *fre* and pET32a(+)Δ*trxA*.

Reagents	**Reaction**[b]									
	1a	**1b**	**2a**	**2b**	**3a**	**3b**	**4a**	**4c**	**5a**	**5b**
Molar ratios (insert: vector)	*3:1*	*9:3*	*3:1*	*9:3*	*3:1*	*9:3*	*3:1*	*9:3*	*3:1*	*9:3*
Insert: *fre* (ng)	23	69	23	69	23	69	23	69	23	69
Vector: pET32a(+)Δ*trxA* (ng)	56	168	56	168	56	168	56	168	56	168
5X ligation buffer (µL)	4	4	4	4	4	4	4	4	4	4
T4 DNA ligase (1U/µL))	1	1	1	1	1	1	1 µL of dilute ligase (0.1 U/ µL)		1	1
dH_2O (µL)	11	3	11	3	11	3	11	3		
Total volume (µL)	24	24	24	24	20	20	20	20	20	20
Conditions	22°C 5 min	22°C 5 min	22°C 1 hr	22°C 1hr	16°C 16 hr	16°C 16 hr	16°C 16 hr	16°C 16 hr	22°C 22 hr	22°C 22 hr

[b] Molar ratios = 30 fmoles: 10 fmoles or 90 fmoles:30 fmoles.

Same set of ligation reactions were carried out with dephosphorylated vector.

Reactions 1-2 were carried out using the Rapid DNA ligation kit (Fermentas Life Sciences, Cat.# K1422).

Reactions 3-5 were carried using standard T4 DNA ligase (Invitrogen, Cat.# 15224-041).

standard T4 DNA ligase (Invitrogen, Cat. #15224-041) and the Rapid DNA ligation kit (Fermentas Life Sciences, Cat.# K1422). Furthermore, ligation reactions were set-up with pET32a(+)Δ*trxA* that was untreated and treated with Antartic Phosphatase (New England Biolabs, Cat#. M02895). Dephosphorylation was achieved by incubating the digested vector at 37°C for 15 minutes followed by heat inactivation at 65°C for 5 minutes. Then, 5 µL of each resulting ligation reaction was chemically transformed or electroporated into competent DH5α cells. The chemical transformation procedure used was as described by Kazem (5), while electroporation was carried out as outlined by Kartono (4); however, LB, rather than SOC, medium was used to resuspend the transformed cells. Finally, 50 to 200 µL of the transformation reaction was plated on LB agar plates containing 50 µg/mL of ampicillin and incubated at 37°C overnight or longer to select for clones containing the plasmid of interest.

Construction of pET32a(+)Δ*trxA/EmoA*. The *emoA* gene was obtained by digesting 5 µg of pEmoA with EcoRI (Invitrogen, Cat.# 15202-021) and React 3 Buffer (Invitrogen, Cat.# 490004) at 37°C for 90 minutes with thermal inactivation at 65°C for 20 minutes. The digested products were resolved on 1% low melting point agarose gel and a 2400 bp band was cut out and gel purified. The re-circularized pET32a(+)Δ*trxA* vector was also digested with EcoRI (Invitrogen, Cat.# 15202-021) under the same conditions to generate matching cohesive ends. The ligation reactions set up are as outlined in Table 3. Each ligation reaction was then transformed into competent DH5α cells as described previously.

Screening of potential clones. To ensure colonies that grew on the LB plates containing 50 µg/mL of ampicillin were not satellite colonies, they were picked and inoculated into 1 mL of LB broth with 100 µg/mL of ampicillin and grown overnight with shaking at 37°C. Selection in broth can possibly select for resistant strain that spread throughout the culture; however, it was performed due to the small size of colonies obtained. The cultures were then plated on LB plates containing 100 µg/mL of ampicillin and incubated at 37°C overnight. Individual colonies were then picked, resuspended in 15 µL distilled water, and mixed with equal volume of cracking buffer (made fresh immediately before use, 0.9 mL of 2X cracking buffer, which consists of: 0.5 mL of 0.5 M EDTA (pH8.0), 0.25 g SDS, 0.0125 bromophenol blue, 3.5 mL glycerol, and made up to 22.5 mL with distilled water; and 0.1 mL of 1M NaOH). The mixture was then incubated at room temperature for 5 minutes, heated at 65°C for 10 minutes, and loaded onto a 1% agarose gel with 1X TBE (M. Boutin, personal communication). A quick plasmid mini-prep using the TENS method (10) was performed on the remaining

Table 3. Conditions for ligation reactions of *emoA* and pET32a(+)Δ*trxA*.

Reagents	**Reaction[c]**							
	6a	**6b**	**7a**	**7b**	**8a**	**8b**	**9a**	**9c**
Molar ratios (insert:vector)	*3:1*	*9:3*	*3:1*	*9:3*	*3:1*	*9:3*	*3:1*	*9:3*
Insert: *emoA* (ng)	64	192	64	192	64	192	64	192
Vector: pET32a(+)Δ*trxA* (ng)	50	150	50	150	50	150	50	150
5X ligation buffer (µL)	4	4	4	4	4	4	4	4
T4 DNA ligase (1U/µL))	1	1	1	1	1	1	1 µL of dilute ligase (0.1 U/ µL)	
dH_2O (µL)	12	6	12	6	8	2	8	2
Total volume (µL)	24	24	24	24	20	20	20	20
Conditions	22°C 5 min	22°C 5 min	22°C 1 hr	22°C 1 hr	4°C 72 hr	4°C 72 hr	16°C 16 hr	16°C 16 hr

[c] Molar ratios = 30 fmoles: 10 fmoles or 90 fmoles:30 fmoles.

Same set of ligation reactions were carried out with dephosphorylated vector.

Reactions 6-7 were carried out using the Rapid DNA ligation kit (Fermentas Life Sciences, Cat.# K1422).

Reactions 8-10 were carried using standard T4 DNA ligase (Invitrogen, Cat.# 15224-041).

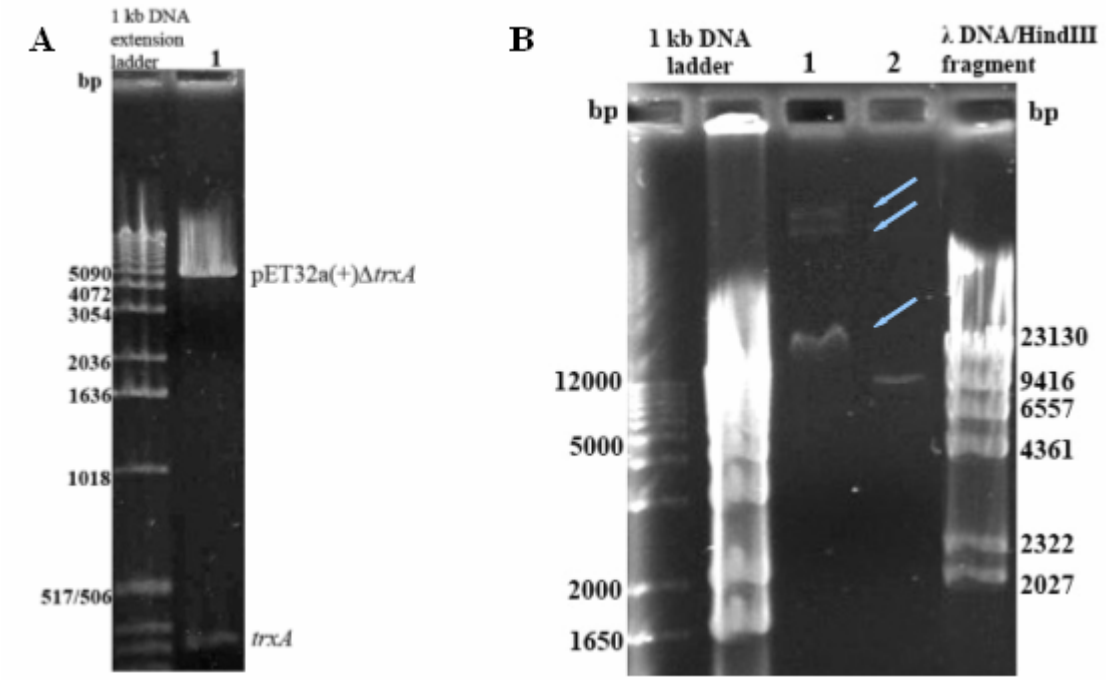

FIG 1. A. Restriction digest analysis of pET32a(+).Lane 1-2: pET32a(+) digested with NdeI to remove *trxA* (320 bp) to yield a 5600 bp fragment, which corresponds to pET32a(+)Δ*trxA*. **B.** Check for ligation of pET32a(+)Δ*trxA* (lane 2) and the control ligation with λ DNA/Hind*III* fragments (lane1).

cultures to isolate DNA that was then digested with EcoRI (Invitrogen, Cat.# 15202-021) at 37°C for 90 minutes and heat inactivated at 65°C for 20 minutes.

RESULTS

Construction of pET32a(+)Δ*trxA*. The pET32a(+) vector was successfully isolated from *E. coli* DH5α cells using the plasmid mini-prep spin column, 2.5 µg of DNA having a A_{260}/A_{280} ratio of 1.8 was obtained. Upon digestion with NdeI, two fragments were produced of size 5600 bp and 320 bp, which corresponded to pET32a(+)Δ*trxA* and *trxA*, respectively (Fig. 1A, lane 1).

The resulting pET32a(+)Δ*trxA* was re-circularized with diluted T4 DNA ligase at 16°C for 16 hours. The ligation was confirmed by agarose gel analysis of isolated DNA. A band of 9500 bp was seen that corresponds to the circular form of the vector (Fig. 1B, lane 2). A positive ligation control, consisting of λ DNA/Hind*III* fragments, was included to confirm that the ligation reaction worked. Several faint high molecular weight bands (Fig. 1B, blue arrows) were obtained representing various fragments of λ DNA/Hind*III* fragments that had been ligated.

The successful ligation of the vector was further verified by transformation into DH5α competent cells. No colonies were obtained from chemically transformed cells; however, with electroporation, 1 colony (SW057) was found. The clone was then screened by performing EcoRI digestion and a 5500 bp fragment was found, which is the expected size of linear pET32a(+) with *trxA* removed (Fig. 2, lane 1).

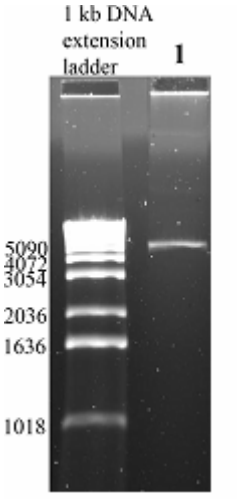

FIG 2. Restriction digest analysis of SW057. EcoRI digest of clone SW057, containing recircularized pET32a(+)Δ*trxA* (lane 1), resulted in a single band of 5500 bp that is of the expected size of linear expression vector without its TrxA tag.

Amplification of *fre* and construction of pET32a(+)Δ*trxA/fre*. Plasmid mini-prep of pES1 isolated 3µg of DNA having a A_{260}/A_{280} ratio of 1.72. The primers and PCR conditions used successfully amplified *fre* from pES1 and an 810 bp fragment was obtained (Fig. 3A, lane 1). When the PCR product was digested with NdeI, two putative *fre* products of approximately 800 bp and 710 bp in size was obtained (Fig. 3B, lane 1). However, only the 800 bp fragment yielded any colonies from electroporation of products that had been ligated with untreated vector at 16°C for 16 hours with diluted T4 DNA ligase.

Colonies were obtained with ligation reactions containing 9:3 insert:vector ratio. DNA from six resulting clones were isolated and screened by EcoRI restriction enzyme digest (Fig. 4, lane 1-6). Out of the six potential clones, clones SW051, SW054, and SW055 had a fragment that ranged in size from 9500 bp to 8300 bp that may correspond to pET32a(+)Δ*trxA*/fre.

Construction of pET32a(+)Δ*trxA/EmoA*. Up to 14 µg of pEmoA plasmid having a A_{260}/A_{280} ratio of 1.9 was effectively isolated from *E. coli* cells. Furthermore, a 2300 bp *emoA* fragment was successfully isolated from pEmoA upon digestion with EcoR1 (Fig.5, lane 1). Despite several attempts with different ligation conditions attempted, no colonies were obtained.

DISCUSSION

The pET32a(+) expression vector contains the *ap* gene that confers resistance to ampicillin (Fig. 6). Thus, after the removal of the *trxA* tag from the vector, only re-circularized pET32a(+)Δ*trxA* that was successfully transformed into *E. coli* DH5α will permit the cells to grow on LB plates containing ampicillin. Furthermore, a single band of approximately 5500 bp was obtained from DNA isolation of clone SW057.

Only one clone was found from this ligation reaction and a plausible cause was the use of NdeI for the removal of the *trxA* tag. A positive ligation control of uncut pET32a(+) was included with each reaction to ensure the viability of the competent cells and to evaluate the transformation method. With chemical and electrocompetent cells the positive control yielded numerous colonies. The single clone was obtained through electroporation, which is several folds more efficient than chemical transformation. Furthermore, low ligation efficiency has been observed and is a common problem in cloning experiments that use NdeI as a restriction enzyme
for digestion (3).

In spite of this, it was found that it was easier to re-circularize the vector than to insert a fragment into the vector. All clones obtained from the construction of pET32a(+)Δ*trxA/fre* contain the 5500 bp fragment of

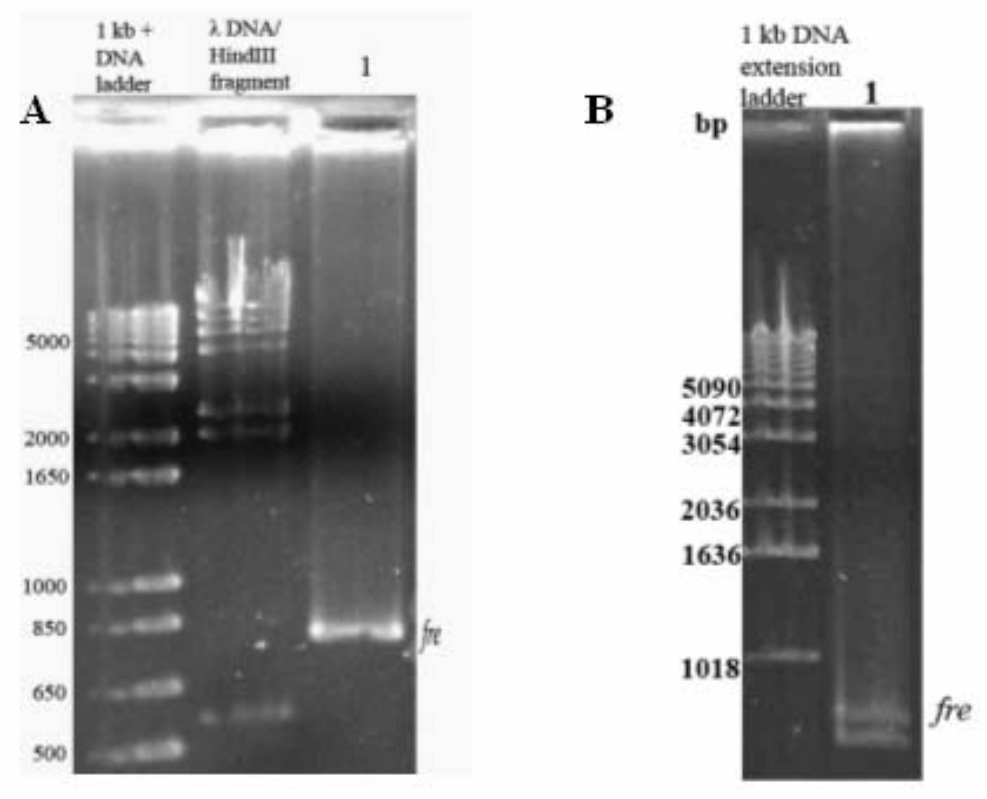

FIG 3. A. PCR amplification of *fre* from pES1 generated an 810 bp fragment. **B.** NdeI digest of PCR amplified *fre* resulted in two fragments 800 bp and 710 bp. The former band is believed to be the correct fragment of flavin oxidoreductase.

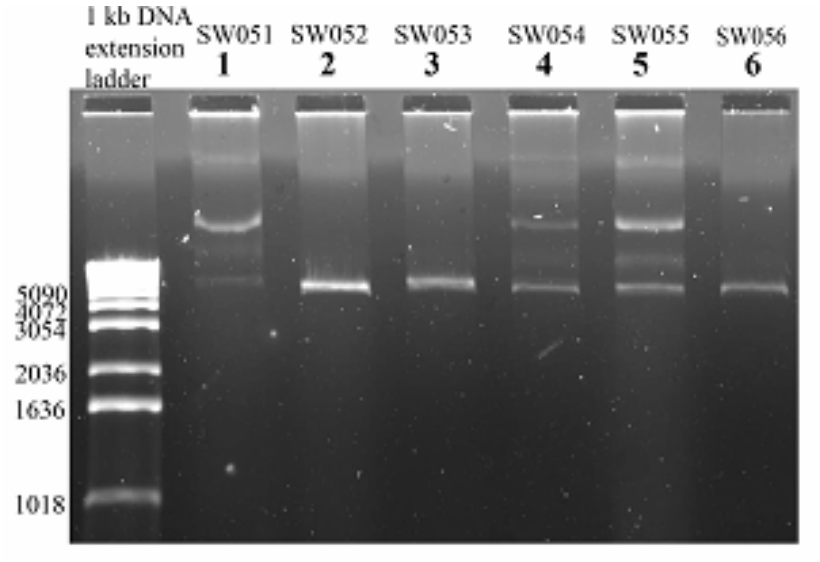

FIG 4. Restriction enzyme digests analysis of potential positive clones for insertion of *fre* into pET32a(+)Δ*trxA*. Lane 1-6: Clones SW051 to SW056 nicked with EcoRI. All lanes contain a 5500 bp band corresponding to ligated pET32a(+)Δ*trxA*. Insertion of *fre* into pET32a(+)Δ*trxA* is seen in clones SW051, SW054, and SW055 (lanes 1, 4, and 5). The pET32a(+)Δ*trxA/fre* fragment ranges in size from 9500 bp to 8300 bp.

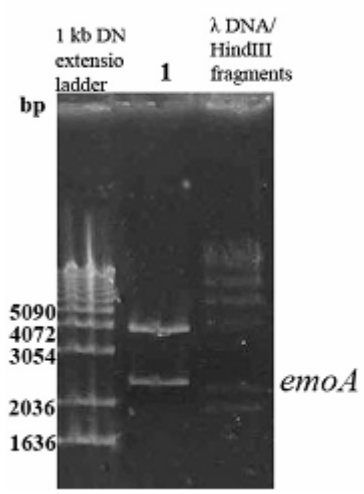

FIG 5. Restriction enzyme digest of pEmoA with EcoRI yielded *emoA* a 2300 bp fragment of expected size (lane 1).

the linear form of the self-ligated vector. This may be due to the fact that the cohesive sites within the vector are close together and the DNA strand can easily fold to join itself together with the help of the T4 DNA ligase. Whereas for joining an insert to a vector, their ends must come into close contact with each other before any bond formation can occur. The probability of getting the close contact was maximized by using a high molar ratios of insert:vector (3:1 and 9:3). In addition, to minimize self-ligation, the vector was treated with Antartic Phosphatase, but no colonies were obtained from ligation reactions with dephosphorylated vector.

Attempts to clone *fre* into pET32a(+)Δ*trxA* yielded three clones that may have the insert. Plasmid clones SW051, SW054, and SW055 have a fragment that ranges in size from 9522 bp to 8299 bp, which is close to the expected size of the 2330 bp *fre* fragment inserted into the 5570 bp vector. The fragments range in size because they were resolved on a 1.5% agarose gel that resulted in poor separation of high molecular weight bands. Clones SW051, SW054, and SW055 also have a high molecular weight band of approximately 15058 bp in size. This large fragment might contain two *fre* inserts ligated into the vector. To confirm that the clones contain the *fre* insert, a double digestion with NdeI and PflMI should be performed and four bands are to be expected. NdeI would remove *fre* from the vector and PflMI would cut the insert into a 290 bp and a 490 bp fragments as well as cutting the vector into two other fragments.

Due to time limitation and low plasmid yield of vector DNA, cloning of *emoA* was not successful. As pET32a(+)Δ*trxA* was isolated using the TENS methods (10), which gave low quality DNA contaminated with proteins, it is not sure whether the ligation reactions did not work due to ligation conditions or due to incomplete or poor digestion of the vector. Proper DNA isolation using commercial columns would assist in obtaining higher yields of better quality DNA for proper EcoRI digestion and evaluation of ligation conditions. The vector can also be digested for a longer period to ensure complete digestion and cleaned-up by performing phenol/chloroform DNA precipitation prior to ligation.

From all the ligation conditions tested, it appears that ligation is most efficient when using low amounts of T4 DNA ligase and the reaction carried out overnight at 16°C. Using these conditions pET32a(+)Δ*trxA* was successfully re-circularized and pET32a(+)Δ*trxA/fre* possibly constructed when using an insert to vector ratio of 9:3. Rapid ligation conditions using high amounts of T4 DNA ligase did not improve the low ligation efficiency of NdeI digested fragments. Although, they might be very effective for the cloning of *emoA* into pET32a(+)Δ*trxA*.

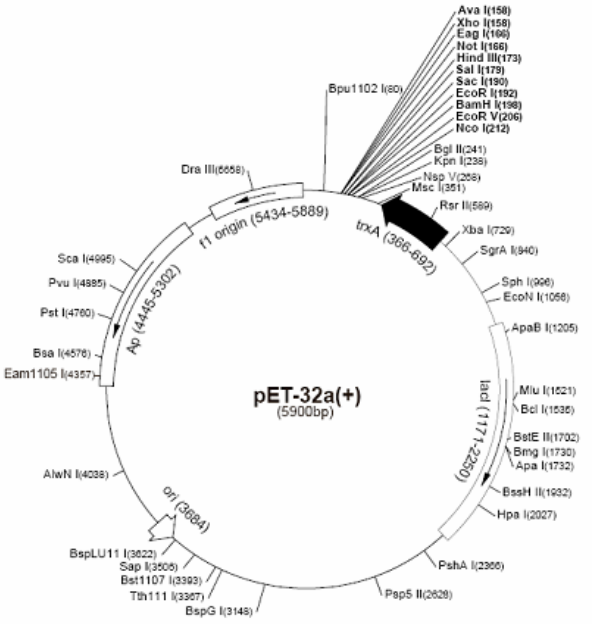

FIG 6. The pET32a(+) expression vector from Novagen. The plasmid confers resistance to ampicillin and has the *trxA* tag, which is flanked by two NdeI restriction sites.

FUTURE EXPERIMENTS

Further restriction enzyme digest analyses need to be performed on the pET32a(+)Δ*trxA/fre* clones to confirm that they contain the insert. Subsequently, the putative clones need to be sequenced to confirm that *fre* has been ligated into the pET32a(+)Δ*trxA*. Then, EmoA can be cloned into the expression vector to study fusion protein properties. The ligation conditions attempted

for the cloning of *emoA* into pET32a(+)Δ*trxA* could be carried out once more with newly isolated pET32a(+)Δ*trxA* from clone SW057. Once the remaining constructs have been made they can be transformed into the expression host *E. coli* BL21(DE3). Protein expression conditions will need to be optimized and assessed for relative protein solubility.

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